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Signature
Date 04/19/06
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Date 04/19/06

TXR No.: 0052059

STUDY TYPE: *In vitro* mammalian chromosome aberrations in human lymphocytes; OPPTS 570.5375 [84-2]; OECD 473.

DP BAR CODE: D313732 (parent, 310098).

P.C. CODE: 090205 TOX. CHEM. NO.: None (new a.i.).

TEST MATERIAL (PURITY): LGC-30473 (Lot No.: P980622, 99% a.i.).

SYNONYM(S): Chemically, *(RS)-N*-(α-cyano-2-thenyl)-4-ethyl-2-(ethylamino)-1,3-thiazole-5- carboxamide; ethaboxam (common name).

CITATION: Allais, L. and Rix, J. (2001). LGC-30473: *In Vitro* Mammalian Chromosome Aberration Test in Human Lymphocytes, performed at Huntingdon Life Sciences Ltd.'s Research Laboratory, Cambridgeshire [ENGLAND]. Project ID: LKF 039/013151, dated 24 October 2001. MRID 46378531. Unpublished.

SPONSOR: LG Chemical, Ltd., Research Park, Taejon [KOREA].

SUBMITTER: LG Life Sciences, c/o Landis International, Inc., Valdosa [GEORGIA].

EXECUTIVE SUMMARY:

In independent *in vitro* mammalian chromosome aberration (metaphase analysis) assays (MRID 46378531), human lymphocytes, stimulated to divide by exposure to phytohemmaglutinin, were treated for 3 hours followed by 16 hours recovery with LGC-30473 (Lot No. P980622, 99% a.i., dissolved in dimethyl sulfoxide, DMSO) at concentrations ranging from 15.6 to 2000 *ug*/mL in the presence and absence of exogenous metabolic activation ±S9 (Trial 1); or continuously for 19 hours at a range of 8 concentrations, 20 to 600 *ug*/mL -S9, and for 3 hours followed by a 16 hour recovery period at the same concentration range +S9 (Trial 2). In addition to cultures exposed to the solvent (representing the "negative" control), other cultures were treated with the clastogenic mutagens, mitomycin C (MMC: 0.1 *ug*/mL for the 19-hour exposures; 0.2 *ug*/mL for the 3-hour exposures) and cyclophosphamide (CPP, 6 *ug*/mL). Two hours before harvest, all cultures were exposed to the anti-mitotic alkaloid, colchicine (as Colcemid®), which arrests cell division at the metaphase stage. Following this treatment, cytotoxicity was determined by Mitotic Index (MI), and the proportion of cells with chromosome aberrations recorded.

In the human lymphocyte cytogenetics assay (MRID 46378531), ethaboxam induced significant ($p < 0.01$) increases in chromosome aberrations and a marked increase in the mitotic index (MI)

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at a concentration of 250 *u*g/mL –S9 after a 3-hour exposure and at 100 *u*g/mL after a 19-hour continuous exposure. The most frequently observed aberration was chromatid breaks suggesting a cytotoxic effect; this observation is supported by the severe cytotoxicity reported as necrotic cells and a reduction in scoreable metaphass at higher concentrations (≥ 500 *u*g/mL, 3-hour exposure; ≥ 200 *u*g/mL –S9, 19-hour exposure). With S9-activation, no firm conclusion can be reached because the levels showing significant increases ($p < 0.01$) in chromosome aberrations (125 and 250 *u*g/mL +S9) were not evaluated in the repeat because of severe cytotoxicity at ≥ 200 *u*g/mL; no explanation was given for excluding 100 *u*g/mL +S9 from testing. Similarly, no explanation was presented for the marked increases in the MIs of cells treated with concentrations as low as 20 *u*g/mL –S9 (626% *vs.* 100% for the solvent control) or 60 *u*g/mL +S9 (145% *vs.* 100% in the solvent control). In agreement with the earlier nonactivated findings, chromatid breaks were the most frequently observed structural aberration.

This study is classified as **unacceptable/guideline,** and does not satisfy the requirements of FIFRA Test Guideline (for *in vitro* cytogenetic) data.

COMPLIANCE: Signed and dated GLP, Quality Assurance, and Data Confidentiality statements were provided.

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I. MATERIALS AND METHODS:

A. MATERIALS:

1. Test Material: LGC-30473,
Description: White powder.
Lot/Batch No.: P980622.
Purity: 99% a.i.
Stability of compound: Not provided.
Solvent used: Dimethyl sulfoxide (DMSO).
Other comments: None.

2. Control Materials:
Negative: None.
Solvent/final concentration: DMSO/1%.
Positive: Non-activated (concentrations, solvent):
Mitomycin C (MMC, 0.1/0.2 *ug*/mL in sterile "purified" water).
Activated: (concentrations, solvent): Cyclophosphamide (CPP, 6 *ug*/mL in sterile "purified" water).

3. Metabolic Activation:

S9 (presumably developed in-house, but not otherwise stated) was derived from hepatic mixed function oxidase homogenates isolated from young (7 to 8 weeks) male Sprague-Dawley rats (< 300 g) stimulated with Aroclor 1254.

x	Aroclor 1254	x	induced	x	rat	x	liver
	phenobarbital		non-induced		mouse		lung
	none				hamster		other
	other						other

4. Describe S9-mix composition (also assumed to be prepared in-house).

Component	**Amount/ Concentration**
S9 fraction	10% v/v
$MgCl_2$	8 mM
KCl	33 mM
Sodium phosphate buffer, pH 7.4	100 mM
Glucose-6-phosphate	5 mM
NADP	4 mM

5. Test Compound Concentrations Used:

a. Preliminary Cytotoxicity Test: (No preliminary cytotoxicity test was performed; MIs were measured in each of the Trials as indicators of cytotoxicity.)

b. Main Chromosome Aberration Assays:

Trial 1:

(1) MI: 20, 40, 60, 80, 100, 200, 400, 600 *u*g/mL ±S9.
(2) Aberrations: 125, 250, 2000 *u*g/mL ±S9.

Trial 2:

(1) MI: 15.6, 31.3, 62.5, 125, 250, 500, 1000 *u*g/mL ±S9.
(2) Aberrations: (a) 20, 80, 100 *u*g/mL –S9.
(b) 20, 60, 80 *u*g/mL +S9.

6. Test Cells

Blood was collected from a "number" (not stated) of healthy, nonsmoking male donors, pooled and diluted with RPMI Tissue Culture Medium, supplemented with 10% fetal calf serum (FCS), 1 unit/mL heparin, 20 I.U. penicillin, 20 *u*g/mL streptomycin, and 2.0 mM glutamine. Aliquots (0.4 mL blood: 4.5 mL medium: 0.1 mL phytohemagglutinin) were incubated at 37°C for 48 hours, with daily shaking (to keep the cells in suspension).

B. TEST PERFORMANCE:

Trial 1:

1. Cell Preparation:

For the nonactivated series, 50 *u*L aliquots of the appropriate dilution of LGC-30473 were added to one set of duplicate cultures, as indicated above, to measure MI. Fifty *u*L of the solvent control and 0.2 ug/mL of MMC were also added to separate duplicate cultures.

Before treatment of the second set of cultures, 1 mL medium was replaced with 1 mL S9-mix, followed by 50 mL aliquots of dilutions of the test

article, to provide the same series of final concentrations as above. Fifty *u*L DMSO and 6 *u*g/mL CPP were also added to duplicate cultures.

Three hours after dosing, all cultures were centrifuged (500 g for 5 minutes), the cell pellets re-suspended in fresh medium, and incubated for another 16 hours.

2. Harvesting and Fixation:

Two hours before harvesting, cell mitosis in each culture was arrested in the metaphase stage by 0.1 *u*g/mL Colcemid®, the cell suspension transferred to a centrifuge tube, and treated as follows:

a. Centrifugation for 5 minutes at 500 g;
b. Cell pellets exposed to hypotonic solution (0.075 M KCl pre-warmed to 37°C for 10 minutes;
c. Centrifugation (500 g for 5 minutes);
d. Cold fixative (3 parts methanol to 1 part glacial acetic acid; repeat fixation until colorless).

3. Slide Preparation:

After a repeat centrifugation, cells were suspended in a small volume of fresh fixative, and dropped onto standard microscope slides. Slides were allowed to air-dry, and then stained in 10% Giemsa; rinsed in buffered water; left to air-dry and finally sealed with DPX.

4. Microscopic Examination:

Under low-power light microscopy, the proportion of mitotic cells per 1000 cells counted in each culture, MI, and the concentration of the test article producing a 50% decrease in MI relative to the solvent control was selected as the highest dose for scoring aberrations; or, failing any decrease, the maximum concentration of 2000 *u*g/mL was chosen as the highest concentration to be tested (HCT). Intermediate and low concentration levels were also selected for examination of metaphase aberrations.

After the slides were coded, metaphase cells were identified under a low power objective, and examined for aberrations using a 1000X oil immersion objective. One hundred metaphases were examined from each culture, and aberrations scored (but only in cells with 44 to 48 chromosomes), according to the classification of the ISCN (1985)[1]. In addition, any polyploid and/or endoreduplicated cells were determined

[1] ISCN (1985). *An International System for Human Cytogenetic Nomenclature*, Harnden, D.G. and Klinger, H.P. (Eds.). S Karper AG, Basel [SWITZERLAND].

quantitatively in the negative control cultures and those treated at the highest concentration of the test article.

5. Trial 2: Cell Cultures:

Cultures were established as described above. In non-activated cultures, treatment was continuous for 19 hours, but remained at 3 hours in the presence of S9-mix. Harvest was 19 hours for both parts of Trial 2. As noted above, concentrations of LGC-30473, with/without S9-mix, were: 20, 46, 60, 80, 100, 200, 400, and 600 *u*g/mL in duplicate. Two cultures were administered the solvent control, whereas, 0.1 *u*g/mL MMC and 6 *u*gCPP were added to separate duplicate cultures. Three hours after dosing, the S9-mix cultures were centrifuged, cell pellets rinsed, re-suspended in fresh medium, and incubated for a further 16 hours.

Two hours before harvest, all cultures were exposed to 0.1 *u*g/mL Colcemid®, then harvested, fixed, and slides prepared and examined as above.

6. Statistics:

The number of aberrant metaphase cells in each treatment group was compared with the solvent control value using Fisher's Exact Test at $p < 0.01$.

7. Assessment of Results:

Criteria for assay acceptance and evaluation of response were both presented. A two-year summary of the laboratory's historical negative and positive control data was also provided, in bar-graph form (MRID 46378531, pp. 30, 31 — APPENDICES 1 AND 2).

II. REPORTED RESULS:

A. **Chemical Analysis:**

The test material was soluble in DMSO at 600 mg/mL. An addition of 1% volume to the aqueous tissue culture medium resulted in compound precipitation at ≥ 1000 *u*g/mL. Accordingly, the highest concentration selected for the cytogenetic evaluation was 2000 *u*g/mL.

B **Preliminry Cytotoxicity Test**: (Not performed.)

C. **Main Cytogentic Assays**:

Trial 1:

Whereas, LGC-30473 caused no reduction in MI at any dose level ±S9, it produced a concentration-related statistically significant increase in MI at 250, 500 and 1000 *ug*/mL (147, 173, and 321%, respectively, compared to 100% for the solvent control) accompanied by increasing numbers of necrotic cells, but no scoreable metaphases were obtained at the highest concentration tested, 2000 *ug*/mL. There were no statistically significant increases in the numbers of polyploid cells by quantitative analysis (MRID 46378531, p. 22, 23 — ATTACHMENT Table 2).

Compared to the solvent control, a statistically significant increase in the proportion of metaphases with chromosomal aberrations was observed at 125 and 250 *ug*/mL ($p < 0.01$), but only when "gaps" were excluded in non-activated culltures treated for 3 hours

Under S9-activation, statistically significant increases in aberrant metaphases, compared to solvent values, were noted at 125 and 250 *ug*/mL of the test article (again, $p < 0.01$, and excluding gaps). In either the absence or presence of S9-activation, the predominant type of scored aberration was chromatid breaks. Both positive controls caused marked statistically significant ($p < 0.001$) in the proportion of aberrant metaphases.

Trial 2:

A drastic reduction in MI (to 33%) occurred at 200 *ug*/mL, following the 19-hour treatment in nonactivated cultures, compared to the solvent control value (MRID 46378531, pp. 26, 27 — ATTACHMENT Table 4). However, the test compound caused significant increases in MI at levels of ≥ 20 *ug*/mL. Few necrotic cells were seen at 80 and 100 *ug*/mL, and an increasing number at ≥ 200 *ug*/mL. In agreement with the earlier fndings, chromatic breaks were the most frequently scored aberration.

Under S9-activation, a dose-related increase in the MI with decreasing dose was seen at 20 g/mL (626% MI) vs 100% for control) to 100 *ug*/mL (265% MI); at these levels, only a few necrotic cells were observed. The quantitative analysis for polyploid clls showed no statistically significant increases in aberrant cultures at any concentration level.

Based on the above findings, concentrations of 20, 60 and 80 g/mL were selected for chromosone analysis. No rationale was given for not selecting 100 ug/mL as the starting concentration for future analysis.

When compared to solvent control values, the test compound caused statistically significant increases in aberrant metaphases at the top concentration, 100 *ug*/mL ($p < 0.01$ with exclusion of gaps) in nonactivated cultures treated for 19 hours, but no increase in aberrations in S9-activated cultures at any concentration level. In agreement with the earlier results, chromatid breaks was the predominant aberration scored. Additionally, both positive controls produced marked increases ($p < 0.001$) in aberrant metaphases.

Therefore, the investigators concluded that LGC-30473 showed evidence of clastogenic activity, but only in the absence of activation, which they suggest could be related to a secondary effect of toxicity

III. REVIEWERS' DISCUSSION/CONCLUSIONS:

A. The EPA reviewers conclude that in the human lymphocyte cytogenetics assay (MRID 46378531), ethaboxam induced significant ($p < 0.01$) increases in chromosome aberrations and a marked increase in the mitotic index (MI) at a concentration of 250 *u*g/mL –S9 after a 3-hour exposure and at 100 *u*g/mL after a 19-hour continuous exposure. The most frequently observed aberration was chromaid breaks suggesting a cytotoxic effect; this observation is supported by the severe cytotoxicity reported as necrotic cells and a reduction in sccoreable metaphases at higher concentrations ($\geq$ 500 *u*g/mL, 3-hour exposure; $\geq$ 200 *u*g/mL –S9, 19-hour exposure). With S9-activation, no firm conclusion can be reached because the levels showing significant increases ($p <$ 0.01) in chromosome aberrations (125 and 250 *u*g/mL +S9) were not evaluated in the repeat because of severe cytotoxicity at $\geq$ 200 *u*g/mL; no explanation was given for excluding 100 *u*g/mL+S9 from testing. Similarly, no explanation was presented for the marked increases in the MIs of cells treated with concentrations as low as 20 *u*g/mL –S9 (626% *vs* 100% for the solvent control) or 60 *u*g/mL +S9 (145% *vs* 100% in the solvent control). In agreement with the earlier nonactivated findings, chromatid breaks was the most frequently observed structural aberration.

B. **Study Deficiencies** : None.